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A Biotechnological Assessment of *Aspergillus Niger*-Produced Pectinase Enzymes' Antibacterial Activity

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Abstract In this study, *Aspergillus niger* was used for the production of pectinase enzymes under submerged fermentation conditions, aiming to evaluate their potential antibacterial activity. Three fungal isolates were tested and optimal conditions for enzyme production were determined regarding incubation time, temperature and pH. The highest enzymatic activity was observed at 40°C and pH 8 after 48 hours of incubation. Quantitative and qualitative assays revealed that pectinase activity ranged between 1.1 and 4.0 U/mg protein, depending on the carbon and nitrogen sources in the growth medium. The antibacterial activity of the produced pectinase was evaluated using the agar well diffusion method against four bacterial strains: *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus* and *Staphylococcus epidermidis*, at concentrations ranging from 4 to 64 µg/mL. The results showed dose-dependent inhibition, with *K. pneumoniae* showing the highest sensitivity (27.33 mm), followed by *E. coli* (19.33 mm), *S. aureus* (15.67 mm) and *S. epidermidis* (6.33 mm). The study highlights the potential of pectinase enzymes from *Aspergillus niger* for antibacterial properties, making them valuable biotechnological agents in medical and industrial applications. These enzymes could be used as natural bio-preservatives or therapeutic agents against resistant bacterial strains. Future research should focus on understanding antibacterial mechanisms, optimizing production and evaluating efficacy in real-world systems.

Key Words *Aspergillus niger*, Pectinase, Antibacterial Activity, Enzyme Biotechnology, Fungal Fermentation

INTRODUCTION

Aspergillus niger is a cosmopolitan representative of microscopic filamentous fungi. Although the main source of this strain is soil, it frequently occurs in various other sources, such as historical and archaeological objects [1].

Pectinases are a group of extracellular enzymes that catalyze the degradation of pectic substances, which are complex polysaccharides found predominantly in the primary cell walls and middle lamella of higher plants [2]. These enzymes include pectin lyase, polygalacturonase and pectin methylesterase, each playing a distinct role in the depolymerization of pectin. Pectinases are widely applied in various industries, such as fruit juice clarification, textile processing, waste treatment and paper manufacturing, due to their ability to modify plant cell wall components efficiently [3]. *Aspergillus niger*, a filamentous fungus, is among the most commonly utilized microorganisms for pectinase production. It has several advantages, including high secretion ability, ease of cultivation and a Generally Recognised as Safe (GRAS) status, which makes it suitable for biotechnological applications. While pectinases are traditionally studied for their industrial benefits, recent investigations have suggested that these enzymes may also exhibit antibacterial properties, especially when produced under specific fermentation conditions [4]. With the rise of antibiotic-resistant bacteria, there is an urgent need to explore novel, natural alternatives to conventional antibiotics. Enzyme-based antimicrobial agents have garnered attention for their biodegradability, specificity and low toxicity. Despite the growing interest, the antibacterial potential of fungal-derived pectinases remains underexplored, particularly concerning their effectiveness against clinically relevant bacterial strains [5].

The present study aims to evaluate the production of pectinase enzymes from *Aspergillus niger* under submerged fermentation conditions and to assess their antibacterial activity against selected Gram-positive and Gram-negative bacteria, including *Staphylococcus aureus*, *Staphylococcus*

epidermidis, *Escherichia coli* and *Klebsiella pneumoniae*. The study further investigates the effect of varying enzyme concentrations on the diameter of inhibition zones, providing insight into the dose-dependent efficacy of these enzymes [6]. By establishing a correlation between enzyme concentration and antimicrobial activity, this research contributes to the growing body of literature supporting the use of fungal enzymes as eco-friendly and effective agents in the fight against bacterial pathogens. The findings may also provide a foundation for future applications of pectinase in food safety, pharmaceuticals and biomedical fields [7].

"Ultimately, advancing the biotechnological production and formulation of *A. niger*-derived pectinase enzymes could contribute to the development of novel, eco-friendly antimicrobial agents, addressing the urgent global need for alternative strategies to combat antibiotic-resistant pathogens."

The primary objectives of this study are:

- To isolate and cultivate *Aspergillus niger* for the production of pectinase enzymes under controlled biotechnological conditions
- To extract, purify and quantify the produced pectinase enzyme for downstream analysis
- To evaluate the antibacterial activity of the *A. niger*-derived pectinase against selected Gram-positive and Gram-negative bacterial strains
- To compare the antibacterial efficacy of pectinase with conventional antimicrobial agents
- To assess the potential of pectinase as a biocontrol or therapeutic agent in combating antimicrobial-resistant (AMR) pathogens

METHODS

Species Isolations

Commercial PDA (potato dextrose agar) was utilized as the culture medium and it was made in the amounts required for the 86 works by the manufacturer's recommendations ("nutrient broth"). The species were sown using this medium in Petri dishes. Following seeding, the 86 plates were incubated for seven days at 35°C to await growth. The spores were transferred to Potato Dextrose agar (PDA) and cultured for four days at 30°C to create the three infected samples that were taken from the Department of Biology at the College of Science. By subculturing the developing colonies on nutrient agar for a long period, a pure culture was produced and the fungal morphology, size, shape and margin were separated on PDA.

Preparation of Strains

Aspergillus niger: To carry out the isolation, an orange that contained the targeted fungus was gathered during the decomposition phase. PDA media was used to cultivate the food pieces, which were then stored at 28°C for seven days. To obtain the isolates, colonies exhibiting the fungus's characteristics as seen under a microscope were selected on a different PDA plate. The species were identified through their macroscopic (colony colour and texture) and microscopic characteristics (Figure 1).

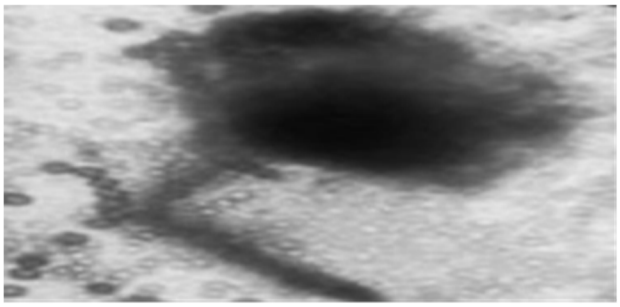

Figure 1: *Aspergillus niger* microscopic visualization from *Aspergillus niger* culture medium Pectinase synthesis and isolation

Liquid Media for Isolation and Production of Tannase

NaNO3 (3 gm), KCl (0.05 gm), MgSo4 (0.05 gm), K2HPO4 (0.1 gm) and containing pectin (1 gm). All components were dissolved in 90 ml, PH was adjusted to 8 and then the volume was completed to 100 mL and sterilized by autoclaving at 121 C for 15 minutes [7].

Solid Culture Media

This medium was prepared with different carbon sources as follows: Wheat barn (10 gm), Tan flour (10 gm), Burglar (10 gm) and Whey (10 gm). Each one of the sources is dissolved in 50 ml of D.W. with 1% tannic acid and adjusted to PH 8 and sterilize by autoclaving at 121°C for 15 minutes [8].

Pectinases Medium for the Semi-Quantitative Method

Potato dextrose agar (6.5 gm), containing pectin (1%). All dissolved in 100 mL of DW, PH was 8 and sterilized by autoclave at 121°C for 15 minutes.

Qualitative Method

With modification, 10 mL of Potato dextrose broth with 1% pectin was inoculated with 0.1 (2.2x104 cells/ml) fungus for 48 h, the enzyme was collected by centrifugation for 15 minutes and enzyme activity was measured in the supernatant [9].

Solution for Measuring Pectinases Activity

Sodium acetate puffer 0.2 M was prepared by dissolving 1.2 gm of sodium acetate in 100 mL of D.W. Adjust ph. = 8, STS (sodium didosalsulphate) 1%, FeCl3 reagent.

Estimation of Pectinases Activity

About 0.5 enzyme with 0.1 mL of tannic acid (0.5%) dissolved in 0.2 M Sodium acetate buffer (PH 8) and incubated at 60°C for 10 minutes, the tubes were centrifuged at 5000 rpm for 10 minutes and the pellet down tannic acid. Pellet was dissolved in 3 mL SDS. Absorbance was measured at 530 nm against a blank after the addition of 1 mL of FeCl3 reagent and a control reaction was done side by side with heat-denatured enzyme. One unit of pectinase activity was defined as the amount of enzyme that hydrolysed 1 mL of substrate tannic acid in 1 minute under the assay conditions [10].

Protein Assay

According to study, precipitation of pectinases by ammonium sulphate. The crude pectinases solution was precipitated with different concentrations of ammonium sulphate (40, 50, 60, 70 and 80%), at saturation under cooling conditions. The precipitant was separated by cooling centrifugation at 8000 rpm for 30 minutes and dissolved in a small amount of phosphate buffer. The solution was dialysed against D.W. of 4°C for 24 h. with many changes of water, 2 h. The activity of the enzyme, protein concentration and specific activity were determined [11].

Determination of Conditions for Pectinases Production

Effect of Incubation Period:100 mL of PDA with 2 mL of activated fungal and incubated at 28°C for different times (24, 48, 72, 96 h), supernatant was assayed for enzyme activity, protein concentration and specific activity.

Effect of Temperature of Incubation

100 mL of PDA with 2 mL of activated fungal and incubated at different temperatures (30, 35, 40 and 45°C) for 48 h. Supernatant was assayed for enzyme activity, protein concentration and specific activity.

Effect of ph

100 mL of PDA with 2 mL of activated fungal at different ph. values (5, 6, 7 and 8) adjusted with 1 N HCl or NaOH, the medium was inoculated with 2 mL of activated fungal suspension and incubated at 30°C for 48 h. Supernatant was assayed for enzyme activity, protein concentration and specific activity [12].

Antibacterial Application of Pectinases Production by Agar Well Diffusion Method

To determine the MIC (minimum inhibitory concentration) of pectinases production on Gram-positive (*Staphylococcus aureus*, *S. epidermidis*) and Gram-negative bacterial strains (*Escherichia coli*, *K. pneumoniae*), the antibacterial activity of the enzyme has been evaluated using the agar-well diffusion method. Isolates were then introduced into nutritional broth and grown at 37°C for 24 hours. The agar diffusion assay was next carried out using Mueller-Hinton agar. Isolates suspensions (0.5 McFarland) onto Muller Hinton agar plates using sterilized cotton swabs. Wells were made using a sterile cork borer and then filled with 100 µL of pectinases production at five different concentrations (64, 32, 16, 8 and 4) µg/mL. The different areas of inhibition were measured again the next day after the plates were incubated for a further 24 hours at 37°C [13].

Data Analysis

The results (from the data obtained) have been shown as Mean±standard deviation of triplicate values. The analysis of the data was carried out using a one-way analysis of variance. Additionally, statistical significance was evaluated using Bonferroni correction at a 95% confidence interval. All statistical analyses were done on Microsoft Excel v. 2016 (Washington, United States).

RESULTS AND DISCUSSION

Three samples were obtained and identified as *A. niger* according to morphology and growth colour on the PDA medium. The result shows that the activity of pectinases in a liquid medium between (1.1-4 U/mg) protein (Figure 2) was cultivated in media containing various nitrogen sources. The result showed that the presence of yeast extract nitrogen source in the medium was the best one for pectinases with a specific activity of 1.1 U\mg protein.

The findings demonstrated that pectinases' activity in solid media ranges from 2 to 3.6 U/mg protein (Figure 3) when they are grown in media that include different types of protein and carbohydrates. According to the results, rice produced the most pectinases with a particular activity. 2.6 U/mg protein, although other sources, such as bulgur, have less specific action. One gram of protein.

Screening for Pectinases Production *Aspergillus niger*

Semi-quantitative screening: PDA medium containing 1% as pectinases was used for screening the pectinases. The results showed that 3 isolates were able to produce pectinases with different zones at 37°C incubation. The ratio (diameter of the clear zone\diameter of colony) ranges between (1-2.2) for isolates grown at 37°C, as shown in Table 1.

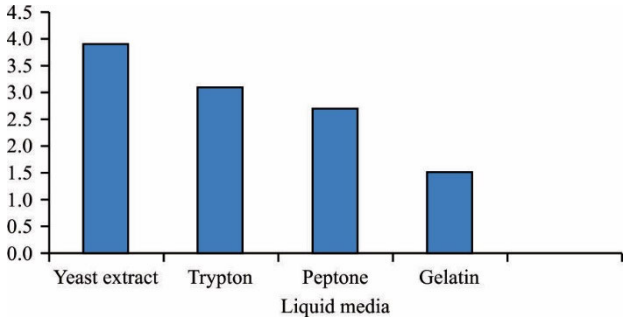

Figure 2: *Aspergillus niger* produces pectinases in solid media using a pH 8 medium that contains 1% pectinases

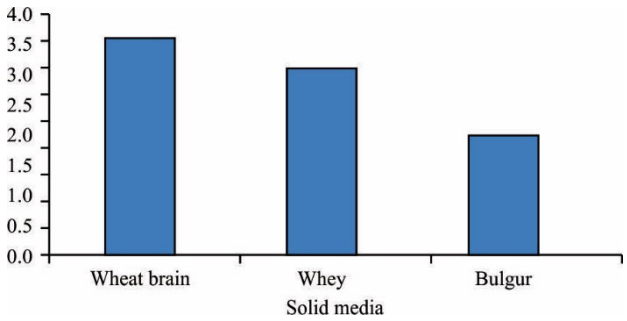

Figure 3: *Aspergillus niger* produces pectinases in a pH 8 medium with 1% pectinases

Quantitative screening: Three isolates (As1, As2 and As3), which have the largest pectinases hydrolysis zone, were selected for quantitative screening of pectinases. The results showed that the activity of pectinases production by these isolates ranges between/1.1-2.1/U/mg protein (Table 2).

Determination of Optimal Conditions for Pectinases Production

Effect of incubation period: The for pectinases production by *Aspergillus niger* was observed after (24, 48, 72, 96 hours) of incubation period. The result in (Figure 4) showed that the production of pectinases started after 24 hour of growth and reached its maximum in 48 hour.

Effected of Temperature of Incubation

pectinases activity was assayed at various incubation temperature (30, 35, 40, 45°C) the result showed that optimal temperature for pectinases production by *Aspergillus niger* was 40°C with specific activity 4 U/mg protein. The decrease of increasing in the incubation temperature leads to a decrease in the enzyme production (Figure 5).

Effected of PH: to investigated the effect of initial pH medium on pectinases production *Aspergillus niger* was grow on PDA medium with the different PH value. The results show that the enzyme production is produced over the ph. range 6-8, with the maximum value of the specific activity 3.5 U/mg protein (Figure 6).

Gram-positive bacteria (*S. aureus, S. epidermidis*) may be more sensitive to pectinase activity than Gram-negative bacteria (*E. coli, K. pneumoniae*). The increase in inhibition zone with higher concentrations supports a dose-dependent antibacterial effect of pectinases. The antibacterial activity of pectinases increased proportionally with enzyme concentration across all tested bacterial strains. At 64 and 32 µg/mL, the enzyme showed the highest inhibition zones, with *K. pneumoniae* exhibiting the greatest susceptibility (27.33 mm), followed by *E. coli* (19.33 mm), *S. aureus* (15.67 mm) and *S. epidermidis* (6.33 mm).

Table 1: *Aspergillus niger* isolates and they're in pectinases agar media incubation at 37°C for 72 hour

Isolation number	Zone of hydrolysis (cm)
As1	1
As2	3.2
As3	2.6

Table 2: Production of pectinases by *Aspergillus niger* isolation in PDB

Number of isolates	Specific activity U\mg protein
As1	1.1
As2	2.1
As3	2

DISCUSSION

The present study aimed to evaluate the antibacterial activity of pectinase enzymes produced by *Aspergillus niger* against

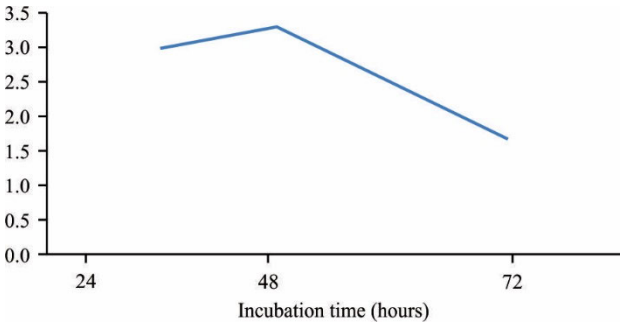

Figure 4: Pectinases production by *Aspergillus* at different times

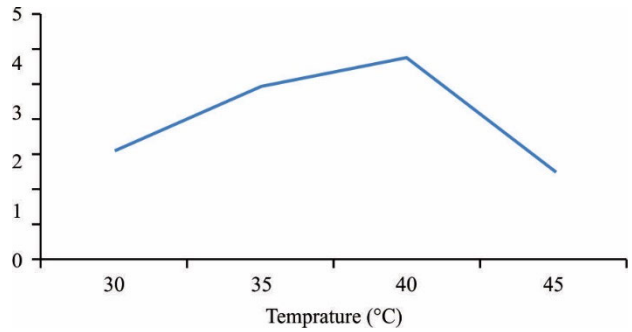

Figure 5: Pectinases production by *Aspergillus* at different temperatures

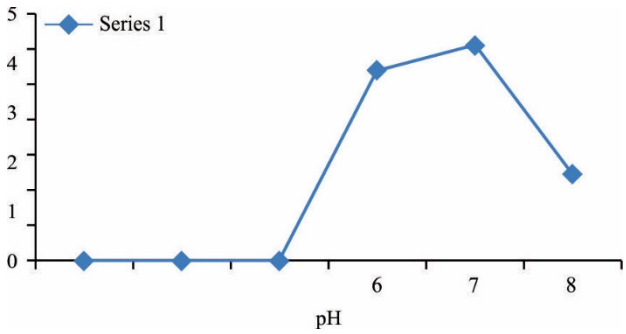

Figure 6: Pectinases production by *Aspergillus niger* at different PH

Table 3: Comparison of inhibition zones of pectinases production among different concentrations in each bacterial species and fungi by unpaired t-test for each concentration and by ANOVA

Bacteria specs	64 µg/ml	32 µg/ml	16 µg/ml	8 µg/ml	4 µg/ml	p-value
E. coli	19.33±1.15	8.33±0.58	0.0±0.0	0.0±0.0	0.0±0.0	<0.001
K. pneumonia pneumoniae	27.33±0.58	14±1.73	0.0±0.0	0.0±0.0	0.0±0.0	<0.001
S. aureus	15.67±0.58	5.67±0.58	0.0±0.0	0.0±0.0	0.0±0.0	<0.001
S. epidermidis	6.33±1.15	5.67±0.31	4.0±0.0	1.5±0.5	0.63±0.55	<0.001
p-value	<0.001	<0.001				

selected Gram-positive and Gram-negative bacterial strains. The results revealed that pectinase exhibits a dose-dependent antibacterial effect, with higher concentrations producing larger inhibition zones.

Among the tested bacterial strains, *Klebsiella pneumoniae* demonstrated the highest sensitivity to pectinase, with a maximum inhibition zone of 27.33 mm at 64 µg/mL. This was followed by *Escherichia coli* (19.33 mm), *Staphylococcus aureus* (15.67 mm) and *Staphylococcus epidermidis* (6.33 mm). These findings indicate that Gram-negative bacteria, particularly *K. pneumoniae*, were more susceptible to the enzymatic activity than the Gram-positive strains, which is somewhat unexpected, as Gram-negative bacteria typically possess an outer membrane that provides additional protection. However, previous studies have reported similar patterns of susceptibility, possibly due to differences in cell wall permeability and enzyme-substrate interaction [14,15].

The mechanism behind the antibacterial effect of pectinase is not yet fully elucidated. It may involve the enzymatic breakdown of pectin-like polysaccharides or glycoproteins in the bacterial cell envelope, leading to structural disruption. Another plausible explanation is that pectinase interferes with bacterial biofilm formation or nutrient acquisition, as noted in earlier reports Gupta and Mandal [8]. Furthermore, the presence of secondary metabolites in the crude pectinase extract may also contribute to its antimicrobial activity [16-18].

The dose-dependent nature of the inhibition zones observed in this study confirms the concentration sensitivity of bacterial response to pectinase treatment. The increase in inhibition zone diameters with increasing enzyme concentrations highlights the potential of fungal enzymes as scalable antibacterial agents, with the possibility of integrating them into novel formulations for pharmaceutical or food- preservation applications [19].

In comparison to synthetic antibiotics, fungal-derived enzymes offer several advantages, including reduced risk of resistance development, biocompatibility and environmental safety [20]. However, further research is necessary to identify the active components responsible for the antibacterial activity, purify the enzyme to homogeneity and evaluate its efficacy in *in vivo* systems or clinical models [21].

Overall, the findings support the hypothesis that pectinases from *Aspergillus niger* exhibit promising antibacterial activity, especially when applied at higher concentrations. These results align with those reported in similar studies [23,24] and further validate the potential of fungal enzymes in biotechnology and medicine [25].

While the antibacterial activity observed in this study is promising, the use of crude enzyme preparations poses several limitations. Crude extracts often contain a mixture of proteins, secondary metabolites and residual media components, which can interfere with or mask the specific activity of pectinase. This lack of purity makes it difficult to attribute observed effects solely to the enzyme itself. Additionally, variability in enzyme concentration and stability in crude extracts may affect reproducibility and accuracy in antibacterial assays. Therefore, further purification and characterization of the enzyme are essential to confirm its specific mechanism of action and potential for clinical or industrial application [26].

CONCLUSIONS

The study shows that *Aspergillus niger*-produced pectinase has antibacterial activity against both Gram-positive and Gram-negative bacteria, suggesting a potential biotechnological application beyond food and textile industries. This enzyme could be used as a natural antimicrobial agent in pharmaceuticals or food systems. However, the use of crude enzyme preparations limits mechanistic insight, necessitating further purification and structural characterization. Future research should explore formulation strategies, synergistic effects with antibiotics and *in vivo* efficacy for eco-friendly alternatives in combating antimicrobial resistance.

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